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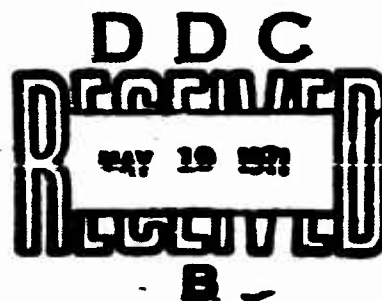
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## Growth of Chikungunya Virus in Baby Hamster Kidney Cell (BHK-21-Clone 13) Suspension Cultures

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This report describes the methods used to obtain high titers of chikungunya virus with suspension cultures of BHK-21-clone 13 cells. The cells were grown at 37°C to a cell concentration of  $10^6$  to  $2 \times 10^6$  per ml. After maximum cell growth, the cells were inoculated with chikungunya virus at a multiplicity of 1 to 2 50% suckling mouse intracerebral lethal doses (SMICLD<sub>50</sub>) per cell in the spent Eagle's minimum essential medium for suspension cultures (MEMS), or the cell cultures were centrifuged at 200 × g and resuspended in either fresh MEMS or medium 199 prior to inoculation. The medium used had no effect on virus titer. The inoculated cultures were incubated at 34°C until the cell viability dropped to 30%, which usually occurred 28 to 30 hr postinoculation. After these procedures, chikungunya virus titers of log<sub>10</sub> 10.3 to 11.8 SMICLD<sub>50</sub> per ml were obtained.

Chikungunya virus was first isolated by Ross (17) during an outbreak of dengue-like fever in Tanganyika, East Africa, in 1953. Subsequent epidemics of dengue-like fever have been recorded in Thailand (15), India (16), Japan (18), and Singapore (8). Although several clinical and epidemiological studies have been made on the virus disease (4-7, 15), no information on the growth of this virus in submerged cell culture is available. A survey of the literature revealed that monolayer cultures of chick fibroblast (9), Vero (10), and FL cells (13) have been used to establish growth curves of the virus. However, optimum parameters, such as cell concentration, multiplicity of infection, and incubation temperatures, have not been established to obtain maximum virus titers.

A preliminary study was made to select a cell line that would grow in suspension and produce high virus titers. It was found from these studies that BHK-21-clone 13 cells (baby hamster kidney fibroblasts) adapted to grow in suspension cultures by using the procedure described by Capstick et al. (2) supported rapid growth and high titers of chikungunya virus. This report defines the procedures used to grow chikungunya virus in suspension cultures of BHK-21-clone 13 cells.

### MATERIALS AND METHODS

**Cell culture.** The cell line used throughout this study was baby hamster kidney (BHK-21-clone 13) initially obtained from the American Type Culture Collection. The origin of BHK-21 has previously been described by Macpherson and Stoker (12).

**Virus seed stock.** A seed stock of chikungunya virus

(Banganké strain) (19) was prepared in the form of a 10% suckling mouse brain suspension in medium 199 containing 5% serum. The seed was stored at -70°C until used.

**Media:** MEM. Eagle's minimum essential medium (MEM) was used to grow BHK-21 monolayers in 8- or 16-oz (240 or 480 ml) prescription bottles. This medium, as described by Merchant et al. (14), consisted of 90% MEM with Hank's balanced salt solution (HBSS) fortified with 2X concentration of L-glutamine and 10% calf serum. Antibiotics were incorporated to give final concentrations per ml of 100 units of penicillin, 100 µg of streptomycin, and 20 units of nystatin.

**Media:** MEMS. Eagle's minimum essential medium for suspension cells (MEMS) was used for growth of cells in suspension; MEM was modified by omission of calcium salts and by a 10-fold increase in NaH<sub>2</sub>PO<sub>4</sub>. Prior to use, 10% by volume of autoclaved Tryptose phosphate broth (Difco) and 10% by volume of filtered calf serum were added. All suspension cultures were grown in the presence of 0.12% methylcellulose of 15-centipoise viscosity. The concentration of antibiotics used was the same as described for MEM.

**Media:** medium 199. Medium 199 was prepared as described by Merchant et al. (14) fortified with 5% calf serum and 0.12% methylcellulose (15 centipoise).

Cells were cultured in suspension in cylindrical Pyrex-jacketed Belco spinners. The volume of culture used (400 ml) occupied about one-half of the vessel. The cultures were stirred by Teflon-coated bar magnets agitated by mechanically propelled external magnets. The cultures were incubated at 37°C.

**Vessels for virus production.** The vessels used for virus growth studies were the Pyrex-jacketed Belco spinners or 1-liter Erlenmeyer shake flasks containing

TABLE 1. Preliminary studies on the yield of chikungunya virus in BHK-21 suspension cells in different media<sup>a</sup>

| Expt | Medium                         | No. of BHK cells per ml | Time (hr) after inoculation | Virus titer (log <sub>10</sub> SMICLD <sub>50</sub> /ml) |
|------|--------------------------------|-------------------------|-----------------------------|--|
| 1    | Spent MEMS plus 10% calf serum | $1.8 \times 10^6$       | 16                          | 8.9  |
|      |                                |                         | 24                          | 10.1   |
|      |                                |                         | 30                          | >10.0  |
|      |                                |                         | 40                          | 8.8  |
|      |                                |                         | 48                          | 9.0  |
|      | Fresh MEMS plus 10% calf serum | $1.8 \times 10^6$       | 16                          | 10.0   |
|      |                                |                         | 24                          | 10.3   |
|      |                                |                         | 30                          | >10.0  |
|      |                                |                         | 40                          | 8.5  |
|      |                                |                         | 48                          | 9.1  |
| 2    | 199 plus 5% calf serum         | $1.14 \times 10^6$      | 21                          | 9.8  |
|      |                                |                         | 24                          | 9.5  |
|      |                                |                         | 28                          | 10.9   |
|      | Fresh MEMS plus 10% calf serum | $1.4 \times 10^6$       | 21                          | 9.7  |
|      |                                |                         | 24                          | 10.1   |
|      |                                |                         | 28                          | 11.2   |

<sup>a</sup> Abbreviations: MEMS, Eagles minimal essential medium for suspension cells; SMICLD<sub>50</sub>, 50% suckling mouse intracerebral lethal dose.

400 ml of medium. All virus-infected cells were maintained at 34°C.

**Adaptation of BHK-21-clone 13 cells to suspension culture.** BHK-21-clone 13 cells that were grown in 8-oz (ca. 240 ml) prescription bottles in MEM at 37°C and had undergone an unknown number of monolayer transfers were used as starter cells for suspension cultures. The monolayer cultures were trypsinized, resuspended in 400 ml of suspension medium at a concentration of  $5 \times 10^5$  cells/ml, and then transferred to the jacketed Bellco spinner vessels. Culture vessels were placed on a Bellco magnetic stirrer at 37°C. During the early stages of adaptation, there was clumping of cells and attachment of the cells to the glass. This was counteracted by trypsinizing the cells with 30 ml of a 5% stock solution of trypsin for each 100 ml of suspension culture. After trypsinization, the cells were removed from suspension by centrifuging for 10 min at  $300 \times g$  and resuspended in fresh medium at a concentration of  $5 \times 10^5$  cells/ml.

In the early stages of adaptation, the generation time was approximately 37 hr; however, after 30 days or 10 complete medium changes the generation time was about 24 hr. After passages of the cells for 30 days in suspension cultures, a cell concentration of  $1.5 \times 10^6$  to  $2.2 \times 10^6$  cells/ml was obtained after a 2-day growth period.

**Cell propagation and virus production.** Cells used for virus production were grown from an initial concentration of  $5 \times 10^5$  cells per ml to a maximum concentration of  $1.5 \times 10^6$  to  $2.2 \times 10^6$  cells per ml in 3 days. The spinner cells were usually trypsinized and centrifuged at  $300 \times g$  for 10 min. When fresh medium was used, the centrifuged cells were resuspended to a cell concentration of  $10^5$  to  $2 \times 10^5$  cells per ml in 400 ml of either fresh MEMS or medium 199. The resuspended cells were transferred to either 400 ml Bellco

spinners or 1,000-ml Erlenmeyer shaker flasks. To each 400 ml of resuspended cells, 0.8 ml of virus seed was added, giving an initial or zero-hour titer of approximately  $2 \times 10^6$  to  $3 \times 10^6$  50% suckling mouse intracerebral lethal doses (SMICLD<sub>50</sub>/ml). The pH of the growth medium was maintained within the range of 7.2 to 7.4 by periodic additions of sterile 5% NaHCO<sub>3</sub> solution.

**Virus assay.** Samples of chikungunya virus were diluted serially 10-fold in Heart Infusion Broth (Difco). For SMICLD<sub>50</sub> titration, 0.03 ml of each dilution was inoculated intracerebrally into 1-day-old suckling mice, 8 mice for each dilution. The LD<sub>50</sub> was

TABLE 2. Effect of number of cells on virus production

| Expt | BHK cell concn/ml in medium 199 | Age of harvest | Virus titer (log <sub>10</sub> SMICLD <sub>50</sub> /ml) |
|------|---------------------------------|----------------|--|
| 1    | $2.5 \times 10^5$               | 24             | 8.7  |
|      | $5 \times 10^5$                 | 24             | 9.6  |
|      | $10^6$                          | 24             | >10.0  |
|      | $2 \times 10^6$                 | 24             | >10.0  |
| 2    | $10^6$                          | 28             | 10.9   |
|      | $2 \times 10^6$                 | 28             | 11.1   |
|      | $3 \times 10^6$                 | 28             | 11.1   |
|      | $4 \times 10^6$                 | 28             | 11.5   |
|      | $10^6$                          | 32             | 10.7   |
|      | $2 \times 10^6$                 | 32             | 10.5   |
|      | $3 \times 10^6$                 | 32             | 10.6   |
|      | $4 \times 10^6$                 | 32             | 11.1   |

<sup>a</sup> SMICLD<sub>50</sub>, 50% suckling mouse intracerebral lethal dose.

TABLE 3. *Virus harvests with standardized BHK-21 cell concentration<sup>a</sup>*

| Expt | Medium                         | No. of BHK cells per ml | Time (hr) after inoculation | Virus titer (log <sub>10</sub> SMICLD <sub>50</sub> /ml) |
|------|--------------------------------|-------------------------|-----------------------------|--|
| 1    | Spent MEMS plus 10% calf serum | $2.1 \times 10^6$       | 30                          | 11.8   |
| 2    | 199 plus 5% calf serum         | $2.1 \times 10^6$       | 28.5                        | 11.8   |
| 3    | 199 plus 5% calf serum         | $2.1 \times 10^6$       | 28.5                        | 11.6   |
| 4    | 199 plus 5% calf serum         | $2.0 \times 10^6$       | 28                          | 11.1   |
| 5    | 199 plus 5% calf serum         | $2.0 \times 10^6$       | 30                          | 10.5   |
| 6    | Fresh MEMS plus 10% calf serum | $2.0 \times 10^6$       | 34                          | 10.4   |
| 7    | Fresh MEMS plus 10% calf serum | $2.0 \times 10^6$       | 34                          | 10.3   |

<sup>a</sup> MEMS, Eagles minimum essential medium for suspension cells; SMICLD<sub>50</sub>, 50% suckling mouse intracerebral lethal dose.

calculated by the Reed and Muench method after a 10-day observation period.

### RESULTS

Preliminary studies on the growth of chikungunya virus in BHK-21 suspension cells in different media are shown in Table 1. The data show that cells in spent MEMS, fresh MEMS, or medium 199 can be used for virus propagation. Samples obtained at designated time intervals showed that maximal titers were obtained approximately 28 to 30 hr postinoculation. Table 2 shows virus yield as a function of number of cells. The data indicate that a cell concentration of  $10^6$  to  $2 \times 10^6$  per ml should be used to obtain high yields of virus. Although the titers obtained by using  $4 \times 10^6$  per ml are higher, it was difficult to maintain the pH of 7.2 to 7.4 throughout the growth cycle. Table 3 shows virus titers of seven trials with a standard cell concentration of  $2 \times 10^6$  per ml. By using either MEMS or medium 199, the trials were harvested when the viability of the cells had dropped to 30%. Assays obtained for these seven trials indicate that chikungunya virus titers of 10.3 to 11.8 log<sub>10</sub> SMICLD<sub>50</sub>/ml can be obtained in 28 to 30 hr in BHK-21 cells.

### DISCUSSION

The above experiments established the feasibility of growing chikungunya virus in BHK-21-clone 13 cell suspension. The virus-rich supernatant fluid, after centrifugation at  $200 \times g$  to remove cells, offers a good starting material for purification and concentration studies because of the low content of nonviral material and high virus titer.

The use of BHK-21-clone 13 cells in suspension cultures provides a simple and efficient method for virus propagation. Handling of cultures is minimal because the medium has sufficient

nutrients for the growth cycle of both the cells and the virus.

Based on the titer of chikungunya virus by the plaque technique as described by Igarashi and Tuchinda (11), the plaque-forming units of a sample were of almost the same degree of magnitude as LD<sub>50</sub> measured by SMICLD<sub>50</sub> titration. A comparison of the virus titers (plaque-forming units) from monolayer cultures of chick fibroblasts (3) or FL cells (13) with titers (SMICLD<sub>50</sub>) obtained from suspension cultures of BHK-21 clone 13 cells demonstrates that higher titers can be obtained from suspension cultures. The data in Table 2 show that virus yield from BHK suspension cultures is directly proportional to the cell number, so a significant increase in virus titer per milliliter is obtained by using high concentrations of BHK cells.

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